The methylotrophic yeast Hansenula polymorpha

van Dijk, R.; Faber, Klaas Nico; Kiel, J.A.K.W.; Veenhuis, M.; van der Klei, I.

Published in:
Enzyme and Microbial Technology

DOI:
10.1016/S0141-0229(00)00173-3

Publication date:
2000

ELSEVIER

Enzyme and Microbial Technology 26 (2000) 793–800

ENZYME and MICROBIAL TECHNOLOGY

www.elsevier.com/locate/enzmictec

The methylotrophic yeast *Hansenula polymorpha*: a versatile cell factory

Ralf van Dijk, Klaas Nico Faber, Jan A.K.W. Kiel, Marten Veenhuis, Ida van der Klei*

Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Postbus 14, 9750 AA Haren, The Netherlands

Received 24 March 1999; received in revised form 19 July 1999; accepted 10 August 1999

Abstract

The development of heterologous overexpression systems for soluble proteins has greatly advanced the study of the structure/function relationships of these proteins and their biotechnological and pharmaceutical applications. In this paper we present an overview on several aspects of the use of the methylotrophic yeast *Hansenula polymorpha* as a host for heterologous gene expression. *H. polymorpha* has been successfully exploited as a cell factory for the large-scale production of such components. Stable, engineered strains can be obtained by site-directed integration of expression cassettes into the genome, for which various constitutive and inducible promoters are available to control the expression of the foreign genes. New developments have now opened the way to additional applications of *H. polymorpha*, which are unprecedented for other organisms. Most importantly, it may be the organism of choice for reliable, large-scale production of heterologous membrane proteins, using inducible intracellular membranes and targeting sequences to specifically insert these proteins stably into these membranes. Furthermore, the use of *H. polymorpha* offers the possibility to accumulate the produced components into specific compartments, namely peroxisomes. These organelles are massively induced during growth of the organism on methanol and may occupy up to 80% of the cell volume. Accumulation inside peroxisomes prevents undesired modifications (e.g. proteolytic processing or glycosylation) and is also in particular advantageous when proteins are produced which are toxic or harmful for the host.

Keywords: Cell factory; Heterologous proteins; Genetic tools; *Hansenula polymorpha*

1. Introduction

Heterologous production of biologically active proteins has become an important tool in fundamental and applied research. At present a variety of hosts (ranging from bacteria to higher eukaryotes) have been explored for their capacity to produce heterologous proteins. In general, microorganisms are favored, because they have short generation times, are easy to grow and are readily accessible to genetic manipulations. Yeasts are often preferred for the production of plant or animal proteins, because in these organisms protein modifications typical for eukaryotes normally occur. These modifications (e.g. glycosylation, acylation, phosphorylation, formation of disulfide bonds; [1]) are often essential for the function and/or stability of the protein. Initially, S*accharomyces cerevisiae* has been the yeast species of choice for foreign protein production for obvious reasons; its genetics were well developed and the organism is generally regarded as save. Despite successful applications specific disadvantages in the use of *S. cerevisiae* have also been encountered. These include among others the oft-observed instability of the engineered strains, undesired hyperglycosylation and relatively low yields, due to the lack of strong promoters.

The methylotrophic yeast *Hansenula polymorpha* has been recognized as an attractive alternative. Originally, methylotrophic yeasts have been isolated for the production of single-cell protein at the expense of cheap carbon sources, like methanol. Studies on the physiology, biochemistry and ultrastructure of these yeasts revealed that methanol is oxidized by a hydrogen-peroxide producing alcohol oxidase (AOX), which is localized in peroxisomes. In these organelles also catalase, which decomposes the hydrogen-peroxide produced, and dihydroxyacetone synthase

* Corresponding author. Tel.: +31-50-3632179; fax: +31-50-3635205.

E-mail address: I.J.van.der.Klei@ biol.rug.nl (I.J. van der Klei).

0141-0229/00/$ – see front matter
PII: S0141-0229(00)00173-3

(DHAS), which catalyzes the first step in the assimilatory pathway, are present. Other enzymes involved in methanol assimilation (enzymes of the xylulose-5-phosphate pathway [2]) or dissimilation [(formaldehyde dehydrogenase (FdDH) and formate dehydrogenase (FMD)] are cytosolic [3].

A striking feature of methanol-grown yeasts is the excessive proliferation of peroxisomes. In methanol-limited continuous cultures these organelles can occupy up to 80% of the total cell volume [3]. In such cells AOX and DHAS may constitute over 60% of the total cell protein, which illustrates that the genes encoding these proteins are controlled by very strong promoters. Also, the expression of the genes encoding FdDH and FMD are under control of strong, inducible promoters [4,5].

H. polymorpha has some specific advantages over other methylotrophic yeasts (*Candida boidinii*, *Pichia pastoris*), being more thermotolerant and capable to grow at higher rates on simple, defined media. The relatively high optimal growth temperature for *H. polymorpha* (37–43°C vs. 30°C for *C. boidinii*, *P. pastoris*, and *S. cerevisiae)* may be favorable for the production of mammalian (including human) proteins and furthermore has the advantage that it allows a better cooling management and reduces the risk of contaminations in large scale fermentations.

In this paper recent relevant developments on the use of *H. polymorpha* as a reliable, efficient cell factory are discussed.

2. Tools for introducing (heterlogous) genes in *H. polymorpha*

2.1. Transformation procedures

The molecular techniques for the manipulation of *H. polymorpha* have now been advanced to a level similar to those available for *S. cerevisiae.* Efficient and reliable transformation procedures for *H. polymorpha* by using electroporation have been developed by Faber et al. [6] (transformation frequency of 1.7×10^6 /μg DNA). Optimal results are obtained by using batch cells from the midexponential growth phase that are pretreated with the reducing agent DTT to weaken the cell wall. Upon addition of plasmid DNA, an electrical pulse is applied that allows entry of the DNA molecules. The electroporation procedure is now the method of choice for efficient *H. polymorpha* transformations.

2.2. Expression vectors

An essential tool for the selection of transformants is the availability of suitable genetic markers. Various auxotrophic *H. polymorpha* mutants (*leu*, *ura*, *trp*, and *ade*) have been generated and vectors have been constructed that carry homologous (*LEU1.1*, *URA3*, *TRP3*, and *ADE11*) or heterologous (*LEU2*, *URA3* from *S. cerevisiae*, *LEU2* from *Candida albicans*) genes that functionally complement these mutants [7–9]. Also, genes that confer resistance against G418 [10], phleomycin [11] or Zeocin [12] have successfully been used as dominant selection markers in *H. polymorpha*.

Several *H. polymorpha* autonomously replicating sequences (HARS) and other DNA-fragments that promote autonomous replication of plasmids in *H. polymorpha* have been isolated and are currently being used in various vectors [8,13–18]. The DNA fragment that contains the *S. cerevisiae LEU2* gene as the selectable marker has also been shown to promote autonomous replication in *H. polymorpha* [18].

Stable transformants, which do not have to be grown under selective growth conditions, are obtained upon integration of plasmid DNA into the genome. For *H. polymorpha* both legitimate and illegitimate integrations have been reported.

Targeted integration can be obtained both by single or double cross-over recombination, resulting in additive integration or gene replacement, respectively. Integration is routinely achieved by using linearized plasmids containing DNA sequences from homologous genes, which are specifically targeted to the corresponding sites in the genome [19]. Most commonly used are the genes encoding alcohol oxidase (*AOX*) or amine oxidase (*AMO).*

An elegant disruption-replacement method for targeted single copy integration has recently been described [20]. First, the *TRP3* gene of an *H. polymorpha leu1.2* strain was disrupted by insertion of the *S. cerevisiae LEU2* gene. The genotype of the resulting strain is *trp3*Δ *LEU2*. The *trp3*Δ allele in this strain and the tightly linked *AOX* gene can subsequently be replaced by a DNA fragment containing part of the *TRP3* gene and expression cassettes containing P_{AOX}. The proper integrants, which are again *TRP3 leu2*, can easily be selected. An attractive advantage of this procedure is that no vector sequences are introduced into the genome of the resulting strains. Moreover, the resulting strain is again *leu1.2*, which offers the possibility to introduce additional genes in the same strain using *LEU2* as selectable marker.

Single- or multicopy integrants can easily be selected by using an *H. polymorpha leu1.1* strain and a vector containing *S. cerevisiae LEU2* as selectable marker. Because multicopy integrants form large colonies on selective media, whereas single-copy integrants develop relatively small ones. The latter is due to the fact that the *S. cerevisiae* gene does not fully complement the leucine auxotrophy in *H. polymorpha.* A similar strategy can be followed by using an *H. polymorpha ura3* strain and the *S. cerevisiae URA3* gene. These strategies have enabled the generation of strains containing more than 100 copies of the vector per genome equivalent [13,21].

Another strategy [22] to select strains with multiple integrations is the use of resistance markers and several consecutive selection rounds by using increasing concentrations of the antibiotic.

2.3. Inducible and constitutive promoters

Like other methylotrophic yeasts, *H. polymorpha* possesses several strong promoters, which are induced during growth on methanol. The promoters of the alcohol oxidase (P_{AOX}), dihydroxyacetone synthase (P_{DHAS}), and formate dehydrogenase (P_{FMD}) genes are most commonly used to control heterologous gene expression [4,5,23,24].

The P_{AOX} promoter is fully repressed by glucose or ethanol and strongly induced by methanol. When glucose or ethanol concentrations are low (e.g. in late exponential batch cultures or carbon-limited continuous cultures), P_{AOX} is derepressed, resulting in low *AOX* expression levels. Derepression also occurs when cells grow in batch cultures containing glycerol or xylitol as sole carbon source. In glycerol or xylitol grown cells the AO levels can reach up to 30% to 70% of the maximal values. The P_{DHAS} and P_{FMD} are also strongly induced during growth on methanol and the kinetics of derepression resemble that of P_{AOX}. These strong regulated promoters are, therefore, tools of choice to control high gene expression levels in *H. polymorpha*.

In constructed strains that are not able to grow on methanol (e.g. upon gene replacement at the AOX-locus) induction of the P_{AOX}, P_{DHAS}, and P_{FMD} can be readily achieved by using continuous cultures and mixed substrates (e.g. glucose/methanol or glucose/choline mixtures). In the latter case the actual inducer is formaldehyde that is released from choline during the metabolism of this nitrogen source [25].

As an independent, inducible promoter the amine oxidase promoter (P_{AMO}) can be used. Amine oxidase (AMO) is a peroxisomal enzyme involved in oxidation of primary amines (methylamine, ethylamine), which can be utilized as sole source of nitrogen by *H. polymorpha*. P_{AMO} is induced by these amines and fully repressed by ammonium [26]. Compared to the P_{AOX} the P_{AMO} is much weaker (up to 20% of P_{AOX}).

Also the promoters of three nitrate assimilation genes, *YNT1*, *YNI1*, and *YNR1*, which are induced by nitrate but strongly repressed by ammonium may serve as additional tools for controlling heterologous gene expression [27]. Furthermore, the recently identified promoter of the *H. polymorpha PHO1* gene encoding a repressible acid phosphatase [28] may be suitable for the control of heterologous genes that have to be expressed at relatively moderate levels.

By using the above promoters various attractive induction strategies can now be designed. For instance, in case of the production of harmful proteins, first biomass can be generated under repressing conditions, followed by an alteration in the composition of the growth medium, thereby inducing the expression of the heterologous gene.

Strong constitutive promoters have been identified in *H. polymorpha* as well. These include the promoters of the genes encoding a plasma membrane ATPase (*PMA1)* [24] or encoding the translation elongation factor-1α (*TEF1/TEF2*; [29] and J.A.K.W. Kiel, unpublished results).

In the course of our studies on the biogenesis of peroxisomes, 10 *H. polymorpha PEX* genes have been cloned, which all encode proteins essential for peroxisome biogenesis. The promoters of the *PEX* genes are relatively weak. Moreover, most of them are constitutively expressed or only slightly induced upon a shift of cells to peroxisome-inducing growth conditions [30–32]. As a consequence these promoters are not very suitable to control heterologous gene expression.

2.4. Sorting of heterologous proteins to specific subcellular locations

By using the above tools, it is possible to introduce foreign genes in *H. polymorpha* and to carefully control their expression. *H. polymorpha* has however an additional attractive advantage that is unprecedented for other cell factories. This includes the possibility to select specific subcellular compartments as optimal targets for specific proteins. Targeting signals have now been identified for various cell compartments of *H. polymorpha* (mitochondrion, peroxisome, endoplasmic reticulum, secretory pathway, vacuole). Such signals can easily be added to any protein, simply by constructing chimeric genes encoding fusion proteins that contain a (homologous or heterologous) targeting signal. If necessary, cleavage of the protein and the signal can be achieved in vivo or in vitro by using highly specific endopeptidases (e.g. the TEV protease [33] or factor Xa [34]) provided that the appropriate cleavage site is introduced between the protein and the signal sequence.

Below some examples are given of the advantage of the sorting the of heterologous proteins to specific target organelles.

2.5. Accumulation in the peroxisomal matrix

Soluble proteins can be specifically targeted to the peroxisomal matrix. These organelles have the capacity to accumulate proteins to very high concentrations. It should be stressed that massive peroxisome proliferation can also be induced under conditions where the organelles are not essential for growth (e.g. in carbon-limited chemostat cultures by using mixed substrates; see above). This implies that the storage capacity of the organelles can in principle be used without affecting the viability of the cell. The advantage of storage in the peroxisomal matrix is the absence of protein modifying enzymes in this cell compartment (e.g. mediating phosphorylation, glycosylation, or proteolytic processing), which may give rise to undesired modifications upon production in the cytosol or during passage in the endoplasmic reticulum. The absence of proteolytic activity in the peroxisomal matrix of *H. polymorpha* is highly advantageous in case of heterologous proteins that are relatively unstable and/or are sensitive to proteolytic degradation.

Accumulation in peroxisomes may also be the method of choice for producing components that are toxic for the host;

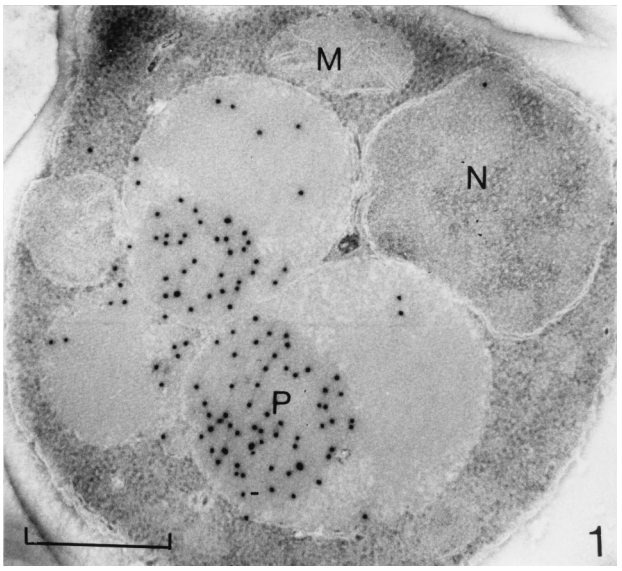

Fig. 1. Immunocytochemical localization of bacterial β-lactamase in peroxisomes of a *Hansenula polymorpha* strain producing PTS1-containing β-lactamase fusion protein (AKL-COOH) [30]. The electronmicrograph shows a thin section of an aldehyde-fixed cell decorated with anti-β-lactamase antibodies and secondairy antibodies conjugated with gold. Abbreviations: M, mitochondrion; N, nucleus; P, peroxisome. The bar represents 0.5 μm.

compartmentalization in peroxisomes will prevent that such components can exert their toxic activity.

Targeting of heterologous proteins to the peroxisomal matrix can be mediated by addition of one of the two known peroxisomal targeting signals, PTS1 or PTS2 [35,36]. The PTS1 consists of only three amino acids and is present at the extreme carboxyterminus of many peroxisomal matrix proteins. The first identified PTS1, -SKL-COOH), was found in firefly luciferase. Later studies revealed that several amino acid substitutions are allowed in this sequence [37]. In *H. polymorpha* the enzymes AOX (-ARF), DHAS (-NKL), catalase (-SKI), and the peroxisomal matrix protein Pex8p (-AKL; see Fig. 1) contain a PTS1, that have been shown to be functional in targeting reporter proteins to peroxisomes of *H. polymorpha*.

The successful use of a PTS1 to accumulate large amounts of a heterologous protein in *H. polymorpha* peroxisomes has been demonstrated [30,38]. The PTS1 (SKL-COOH) was added to the carboxyterminus of a fusion protein, which consisted of human insulin-like growth factor II and a carrier protein. Overproduction of this protein, which was properly targeted to the peroxisomes, resulted in levels of $>$20% of the total cell protein [34].

The PTS2 is present at the N-terminus of peroxisomal matrix proteins and characterized by the consensus sequence (R/K)(L/V/I)X_5(H/Q)(L/A) [35,36]. In *H. polymorpha* this signal has been found in AMO (RLX_5QA) and Pex8p (KLX_5QL). Import of PTS1 proteins is efficient under all growth conditions tested so far. However, for efficient import of PTS2 proteins the cells have to be grown in media containing primary amines as sole nitrogen source (i.e. conditions that induce expression of the *AMO* gene), possibly because only under these conditions the genes encoding essential components involved in PTS2 protein import are sufficiently expressed [18].

2.6. Intracellular membranes

Of special interest is the establishment of expression systems for overproduction of heterologous membrane proteins. Large-scale production of foreign membrane proteins is troublesome in all systems used to date. On the other hand, the production of membrane proteins (e.g. human hormone receptors) is of major importance in fundamental studies for obtaining sufficient amounts of proteins for functional studies or for three-dimensional structure determinations. Membrane proteins form a large and important class of proteins. Analysis of the available eukaryotic genomes shows that at least 30% of the open reading frames encode membrane proteins. Moreover, membrane protein malfunctioning is the cause of many inherited diseases in man. Hence, the availability of a reliable system to overproduce these proteins is of great medical interest, e.g. for rational drug design. *H. polymorpha* can fill in the existing gap in membrane protein production systems. The organism has the advantage that excessive amounts of peroxisomal membranes can be produced that largely lack homologous proteins. These membranes are induced during incubation of cells on oleic acid-containing media (*H. polymorpha* is not capable to grow on oleic acid) [39], but do not form organelles and thus have no matrix contents. Therefore, these membranes are ideal targets to accumulate foreign membrane proteins. Because these membranes are peroxisomal in nature, sorting can be mediated by a specific targeting signal of a homologous *H. polymorpha* peroxisomal membrane protein. Efficient sorting of specific human membrane proteins that were biologically active has already been achieved by using the N-terminus of Pex3p [32] as sorting signal.

2.7. Secretion

Secretion of an (over)produced heterologous protein is often favorable, because it facilitates the recovery of the protein from the culture fluid. Also in this respect *H. polymorpha* offers a distinct advantage, because this yeast species does not secrete significant amounts of proteins. Therefore, the secreted heterologous protein can be recovered in a relatively pure state from the culture medium ($>$90% of total extracellular protein). Another important aspect of protein secretion is that in the secretory pathway of eukaryotes several protein modifications take place, which are often essential for the activation of proteins (proteolytic processing, formation of the correct disulphide bonds, glycosylation etc.).

To establish secretion it is necessary to fuse the protein of interest to an N-terminal presequence (also called leader

sequence) that directs the protein to the secretory pathway. Leader sequences have a tripartite structure, consisting of a positively charged region at the extreme N-terminus, a central hydrophobic stretch of 7 to 15 amino acids, followed by a polar region (3–7 amino acids) that contains the recognition site for a specific ER-located leader peptidase. The cleavage site is characterized by small side chains at position −1 and −3 relative to the residue where the presequence is processed. Some secreted proteins contain, next to the presequence, a proregion. These preprosequences are processed twice. The presequence is cleaved by the ER-leader peptidase, whereas the second one is cleaved in the late Golgi by a functional equivalent of *S. cerevisiae* Kex2 [40,41]. Two basic residues (KR or RR) precede the second cleavage site.

So far only one homologous *H. polymorpha* secretion signal is known, namely the presequence of repressible acid phosphatase (*PHO1).* This presequence is a good candidate to mediate secretion of heterologous proteins in *H. polymorpha* [28], but has not been tested yet.

Of the various heterologous secretion signals available to drive protein secretion in *H. polymorpha*, the preprosequence of *S. cerevisiae* mating factor 1α is mostly used [42,43,44]. As illustrated in Table 1, this signal invariable resulted in efficient secretion in *H. polymorpha*.

Other heterologous prepro-sequences, which have successfully been used in *H. polymorpha* are derived from *Schwanniomyces occidentalis* glucoamylase [5,45], *Carcinus maenas* hyperglycemic hormone [45], *Aspergillus niger* glucoamylase [20], *Kluyveromyces lactis* killer toxin [20], and *Kluyveromyces marxianus* inulinase [46] (see Table 1).

Expression of *S. cerevisiae SUC2* [47], which codes for invertase including its authentic leader sequence, resulted in efficient secretion of Sc-invertase protein by *H. polymorpha* [48]. Also upon fusion of the Sc-invertase presequence to Ct-α-galactosidase (see Table 1), the heterologous protein was successfully secreted. However, when the Sc-invertase leader sequence was fused to human tyroid peroxidase this signal was not effective (Table 1). Experiments in which different leader sequences were compared with respect to their capacity to direct a protein to the secretory pathway of *H. polymorpha*, suggested that the search for the most efficient leader sequence is often a matter of trial and error (Table 1).

Several examples have been described of proteins (ranging from fungal to human ones) that carried the authentic, endogenous presequence and were properly directed to the secretory pathway of *H. polymorpha* and subsequently secreted (see Table 1).

Although the secretory pathway is highly conserved in eukaryotes, not all heterologous targeting sequences function efficiently in *H. polymorpha*.

Proper translation of the presequence could be hampered due to the presence of rare codons for *H. polymorpha* [20] resulting in a decrease in translation rate or even in a translation block. Adjustments in the coding regions can solve such problems. Incorrect or incomplete processing of the pre(pro)sequences can also result in reduced secretion efficiency. Examples in *H. polymorpha* are the secretion of human urinary plasmalogen activator (slow processing of the prosequence) or leech hirudin (incorrect processing resulting in one amino acid extension) [20,45]. These problems are generally related to insufficient recognition of the cleavage site(s) by the processing peptidases. A rational design of pre(pro)sequences and processing sites based on empirical rules as postulated by von Heijne [49], has proven to be effective for *S. occidentalis* preprosequences [45], and could be applied for *H. polymorpha* as well.

Proper glycosylation is another important aspect of the production of functional proteins in heterologous hosts. The

Table 1
Heterologous proteins secreted by *Hansenula polymorpha*

Protein[a]	Secretion signal[b]	Secretion yield[c]	Reference
An-Glucose oxidase	*Sc*-αMF	+++/+++++	43/55
Hs-Kunitz-PI	*Sc*-αMF	+++	56
Aprotinin	*Sc*-αMF	++/+++[d]	11
Sc-Invertase	endogenous	+++++[e]	48
Ct-α-Galactosidase	*Sc*-invertase	+++/+[f]	57/58
Hs-Tyroid Peroxidase	*Sc*-αMF	+++[g]	44
Hs-Tyroid Peroxidase	*Sc*-invertase	−	44
Hs-Tyroid Peroxidase	endogenous	−	44
Hs-α*1*-Antitrypsin	*Km*-inulinase	++++[h]	59
Hm-Huridin	*Sc*-αMF	+++	45
Hm-Huridin	*So*-GAM	++++	45
Hm-Huridin	*Cm*-CHH	++++	45
Hs-Urokinase	*Sc*-αMF	++++	20
Hs-Urokinase	*KI*-KTS	+++++	20
Hs-Urokinase	*An*-GAS	+	20
Hs-Urokinase	*Hs*-OLS	+++++	20
Aa-Cellulase-I	endogenous	+++++	51
Hi-Cellulase-II	endogenous	+++++	51
Aa-Galactanase	endogenous	+++++	51
TI-Lapase I	endogenous	++	51
Aa-Polygalacturonase	endogenous	++	51
HI-Xylanase-I	endogenous	+++++	51
So-Glucoamylase	endogenous	++++	5
HBsAG-M	endogenous	++[e]	60

[a] Abbreviations: *Hs, Homo sapiens*; *Sc, Saccharomyces cerevisiae*; *Aa, Aspergillus aculeatus. Hi, Humicola insolens; Ct, Cyamopsis tetragonoloba. So, Schwannomiomyces occidentalis; An, Aspergillus niger; s, Spinach;* HBsAG-M, Hepatitis B surface antigen middle; *Hm, Hurido medicinalis;* Kunitz-PI, protease inhibiter domain; *TI, Thermomyces lanuginosus.*

[b] Abbreviations: *Km, Kluyveromyces marxianus; Sc*-αMF, *S. cerevisiae* prepro α mating factor; *KI*-KTS, *Kluyveromyces lactis* killer toxin; *An*-GAS, *Aspergillus niger* glucoamylase; *Hs*-OLS, Human lysozyme; *So*-GAM, *Schwanniomyces occidentalis* glucoamylase; *Cm*-CHH, *Carcinus maenas* hyperglycemic hormone.

[c] +–+++++ is from 100 mg/l to at least 1g/l product, otherwise based on activity comparison.

[d] Over 80% extracellular; dependent on the form of the protein.

[e] Periplasmic space.

[f] Deglycosylation necessary for activity.

[g] Not active membrane bound.

[h] Over 80% extracellular.

majority of human therapeutic proteins are glycoproteins, but improper glycosylation of heterologous proteins may cause an immunogenic response. Hence, it is of utmost importance that the glycosylation patterns resemble those present in the authentic proteins.

In *S. cerevisiae*, hyperglycosylation has often been observed (i.e. heterogenous glycosylation by addition of more than 40 mannose residues to the glycosylation core). In general, the overall length of the mannose chains is lower in *H. polymorpha* compared to *S. cerevisiae* and only few cases of hyperglycosylation have been reported in *H. polymorpha* [50,51]. Hence, *H. polymorpha* may be a much better candidate to produce human therapeutic proteins compared to *S. cerevisiae.*

3. Conclusion

At present a range of heterologous expression systems is available to cover the need for various proteins to be used for fundamental structure/function analysis and for biotechnological and pharmaceutical purposes. Among these systems, *H. polymorpha* is now recognized as a very suitable one. The first recombinant products produced in *H. polymorpha* have now passed the clinical examinations and are being launched on the market (e.g. a novel Hepatitis B vaccine) [52,53] and others will soon follow. Also, the use of *H. polymorpha* as a biocatalyst [54] will be extended in the near future in particular when the option to anchor factors involved in the activation of complex heterologous proteins at the periplasmic space or outer cell wall will be realized. This strategy allows the creation of regeneratable systems in continuous cultures.

However, the real challenge for the next coming years is to further develop the high potential of the organism for the production of various membrane proteins (including human). The need for such a system is obvious and would strongly stimulate the functional and structural analyses of this important class of proteins.

Acknowledgments

R.v.D. and I.J.v.d.K. are supported by a grant of the Netherlands Technology Foundation (STW), which is subsidized by the Netherlands Organization for Scientific Research (NWO). K.N.F. is supported by a PULS grant from NWO.

References

[1] Reiser J, Glumoff V, Kalin M, Ochsner U. Transfer and expression of heterologous genes in yeasts other than *Saccharomyces cerevisiae*. Adv Biochem Eng Biotechnol 1990;43:75–102.

[2] van Dijken JP, Veenhuis M, Harder W. Peroxisomes of methanol-grown yeasts. Ann NY Acad Sci 1982;386:200–16.

[3] Veenhuis M, van Dijken JP, Pilon SA, Harder W. Development of crystalline peroxisomes in methanol-grown cells of the yeast *Hansenula polymorpha* and its relation to environmental conditions. Arch Microbiol 1978;117:153–63.

[4] Janowicz ZA, Merckelbach A, Eckart M, Weydemann U, Roggenkamp R, Hollenberg CP. Expression system based on the methylotrophic yeast *Hansenula polymorpha*. Yeast 1988;4S:155.

[5] Gellissen G, Janowicz ZA, Merckelbach A, Piontek M, Keup P, Weydemann U, Hollenberg CP, Strasser AW. Heterologous gene expression in *Hansenula polymorpha*: efficient secretion of glucoamylase. Biotechnology (NY) 1991;9:291–5.

[6] Faber KN, Haima P, Harder W, Veenhuis M, AB G. Highly-efficient electrotransformation of the yeast *Hansenula polymorpha.* Curr Genet 1994;25:305–10.

[7] Agaphonov MO, Poznyakovski AI, Bogdanova AI, Ter–Avanesyan MD. Isolation and characterization of the *LEU2* gene of *Hansenula polymorpha.* Yeast 1994;10:509–13.

[8] Bogdanova AI, Agaphonov MO, Ter–Avanesyan MD. Plasmid reorganization during integrative transformation in *Hansenula polymorpha.* Yeast 1995;11:343–53.

[9] Merckelbach A, Godecke S, Janowicz ZA, Hollenberg CP. Cloning and sequencing of the ura3 locus of the methylotrophic yeast *Hansenula polymorpha* and its use for the generation of a deletion by gene replacement. Appl Microbiol Biotechnol 1993;40:361–4.

[10] Gellissen G, Melber K. Methylotrophic yeast *Hansenula polymorpha* as production organism for recombinant pharmaceuticals. Arzneimittelforschung 1996;46:943–8.

[11] Zureck C, Kubis E, Keup P, Horlein D, Beunink J, Thommes J, Kula M-R, Hollenberg CP, Gellissen G. Production of two Aprotinin variants in *Hansenula polymorpha.* Process Biochem 1996;7:679–89.

[12] Gatignol A, Baron M, Tiraby G. Pleomycin resistance encoded by the ble gene from transposon Tn5 as a dominant selectable marker in *Saccharomyces cerevisiae.* Mol Gen Genet 1987;207:342–8.

[13] Roggenkamp R, Hansen H, Eckart M, Janowicz Z, Hollenberg CP. Transformation of the methylotrophic yeast *Hansenula polymorpha* by autonomous replicating and integrating vectors. Mol Gen Genet 1986;202:302–8.

[14] Sohn JH, Choi ES, Kim CH, Agaphonov MO, Ter–Avanesyan MD, Rhee JS, Rhee SK. A novel autonomously replicating sequence (ARS) for multiple integration in the yeast *Hansenula polymorpha* DL-1. J Bacteriol 1996;178:4420–8.

[15] Sohn JH, Choi ES, Kang HA, Rhee JS, Rhee SK. A family of telomere-associated autonomously replicating sequences and their functions in targeted recombination in *Hansenula polymorpha* DL-1. J Bacteriol 1999;181:1005–13.

[16] Tikhomirova LP, Ikonomova RN, Kuznetsova EN. Evidence for autonomous replication and stabilization of recombinant plasmids in the transformants of yeast *Hansenula polymorpha.* Curr Genet 1986; 10:741–7.

[17] Berardi E, Thomas DY. An effective transformation method for *Hansenula polymorpha*. Curr Genet 1990;18:169–70.

[18] Faber KN, Haima P, Gietl C, Harder W, AB G, Veenhuis M. The methylotrophic yeast *Hansenula polymorpha* contains an inducible import pathway for peroxisomal matrix proteins with an N-terminal targeting signal (PTS2 proteins). Proc Natl Acad Sci USA 1994;91: 12985–9.

[19] Kiel JAKW, Keizer–Gunnink IK, Krause T, Komori M, Veenhuis M. Heterologous complementation of peroxisome function in yeast: the *Saccharomyces cerevisiae* PAS3 gene restores peroxisome biogenesis in a *Hansenula polymorpha* per9 disruption mutant. FEBS Lett 1995; 377:434–8.

[20] Agaphonov MO, Beburov MY, Ter–Avanesyan MD, Smirnov VN. A disruption-replacement approach for the targeted integration of foreign genes in *Hansenula polymorpha.* Yeast 1995;11:1241–7.

[21] Janowicz ZA, Melber K, Merckelbach A, Jacobs E, Harford N, Comberbach M, Hollenberg CP. Simultaneous expression of the S and L

surface antigens of hepatitis B, and formation of mixed particles in the methylotrophic yeast *Hansenula polymorpha*. Yeast 1991;7:431–3.
[22] Gatzke R, Weydemann U, Janowicz ZA, Hollenberg CP. Stable multicopy integration of vector sequences in *Hansenula polymorpha*. Appl Microbiol Biotechnol 1995;43:844–9.
[23] Faber KN, Harder W, AB G, Veenhuis M. Review: methylotrophic yeasts as factories for the production of foreign proteins. Yeast 1995;11:1331–44.
[24] Hollenberg CP, Gellissen G. Production of recombinant proteins by methylotrophic yeasts. Curr Opin Biotechnol 1997;8:554–60.
[25] Zwart KB, Veenhuis M, Harder W. Significance of yeast peroxisomes in the metabolism of choline and ethanolamine. Antonie Van Leeuwenhoek 1983;49:369–85.
[26] Zwart KB, Veenhuis M, Plat G, Harder W. Characterization of glyoxysomes in yeasts and their transformation into peroxisomes in response to changes in the environmental conditions. Arch Microbiol 1983;136:28–38.
[27] Avila J, Gonzalez C, Brito N, Siverio JM. Clustering of the YNA1 gene encoding a Zn(II)2Cys6 transcriptional factor in the yeast *Hansenula polymorpha* with the nitrate assimilation genes YNT1, YNI1 and YNR1, and its involvement in their transcriptional activation. Biochem J 1998;335:647–52.
[28] Phongdara A, Merckelbach A, Keup P, Gellissen G, Hollenberg CP. Cloning and characterization of the gene encoding a repressible acid phosphatase (PHO1) from the methylotrophic yeast *Hansenula polymorpha*. Appl Microbiol Biotechnol 1998;50:77–84.
[29] Baerends RJS, Salomons FA, Faber KN, Kiel JAKW, Van der Klei IJ, Veenhuis M. Deviant Pex3p levels affect normal peroxisome formation in *Hansenula polymorpha*: high steady-state levels of the protein fully abolish matrix protein import. Yeast 1997;13:1437–48.
[30] Waterham HR, Titorenko VI, Haima P, Cregg JM, Harder W, Veenhuis M. The *Hansenula polymorpha PER1* gene is essential for peroxisome biogenesis and encodes a peroxisomal matrix protein with both carboxy- and amino-terminal targeting signals. J Cell Biol 1994;127:737–49.
[31] Van der Klei IJ, Hilbrands RE, Kiel JAKW, Rasmussen SW, Cregg JM, Veenhuis M. The ubiquitin-conjugating enzyme Pex4p of *Hansenula polymorpha* is required for efficient functioning of the PTS1 import machinery. EMBO J 1998;17:3608–18.
[32] Baerends RJS, Rasmussen SW, Hilbrands RE, van der Heide M, Faber KN, Reuvekamp PTW, Kiel JAKW, Cregg JM, Van der Klei IJ, Veenhuis M. The *Hansenula polymorpha PER9* gene encodes a peroxisomal membrane protein essential for peroxisome assembly and integrity. J Biol Chem 1996;271:8887–94.
[33] Carrington JC, Dougherty WG. A viral cleavage site cassette: identification of amino acid sequences required for tobacco etch virus polyprotein processing. Proc Natl Acad Sci USA 1988;85:3391–5.
[34] Faber KN, Westra S, Waterham HR, Keizer–Gunnink I, Harder W, AB G, Veenhuis M. Foreign gene expression in *Hansenula polymorpha*. A system for the synthesis of small functional peptides. Appl Microbiol Biotechnol 1996;45:72–9.
[35] Rachubinski RA, Subramani S. How proteins penetrate peroxisomes. Cell 1995;83:525–8.
[36] Subramani S. Convergence of model systems for peroxisome biogenesis. Curr Opin Cell Biol 1996;8:513–8.
[37] Elgersma Y, Vos A, van den Berg M, van Roermund CW, van der Sluijs P, Distel B, Tabak HF. Analysis of the carboxyl-terminal peroxisomal targeting signal 1 in a homologous context in *Saccharomyces cerevisiae*. J Biol Chem 1996;271:26375–82.
[38] Hansen H, Didion T, Thiemann A, Veenhuis M, Roggenkamp R. Targeting sequences of the two major peroxisomal proteins in the methylotrophic yeast *Hansenula polymorpha*. Mol Gen Genet 1992; 235:269–78.
[39] Veenhuis M, Kram AM, Kunau WH, Harder W. Excessive membrane development following exposure of the methylotrophic yeast *Hansenula polymorpha* to oleic acid-containing media. Yeast 1990;6:511–9.
[40] Zhu YS, Zhang XY, Cartwright CP, Tipper DJ. Kex2-dependent processing of yeast K1 killer preprotoxin includes cleavage at Pro-Arg-44. Mol Microbiol 1992;6:511–52.
[41] Julius D, Brake A, Blair L, Kunisawa R, Thorner J. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor. Cell 1984; 37:1075–89.
[42] Brake AJ. Secretion of heterologous proteins directed by the yeast alpha-factor leader. Biotechnol 1989;13:269–80.
[43] Hodgkins M, Mead D, Ballance DJ, Goodey A, Sudbery P. Expression of the glucose oxidase gene from *Aspergillus niger* in *Hansenula polymorpha* and its use as a reporter gene to isolate regulatory mutations. Yeast 1993;9:625–35.
[44] Wedlock N, Furmaniak J, Fowler S, Kiso Y, Bednarek J, Baumann–Antczak A, Morteo C, Sudbery P, Hinchcliff A, Rees SB. Expression of human thyroid peroxidase in the yeasts *Saccharomyces cerevisiae* and *Hansenula polymorpha*. J Mol Endocrinol 1993;10:325–36.
[45] Weydemann U, Keup P, Piontek M, Strasser AW, Schweden J, Gellissen G, Janowicz ZA. High-level secretion of hirudin by *Hansenula* polymorpha–authentic processing of three different preprohirudins. Appl Microbiol Biotechnol 1995;44:377–85.
[46] Laloux O, Cassart JP, Delcour J, Van Beeumen J, Vandenhaute J. Cloning and sequencing of the inulinase gene of *Kluyveromyces marxianus* var. marxianus ATCC 12424. FEBS Lett 1991;289:64–8.
[47] Sarokin L, Carlson M. Upstream region required for regulated expression of the glucose-repressible *SUC2* gene of *Saccharomyces cerevisiae*. Yeast 1991;7:463–73.
[48] Rodriguez L, Narciandi RE, Roca H, Cremata J, Montesinos R, Rodriguez E, Grillo JM, Muzio V, Herrera LS, Delgado JM. Invertase secretion in *Hansenula polymorpha* under the AOX1 promoter from *Pichia pastoris*. Yeast 1996;12:815–22.
[49] von Heijne G. A new method for predicting signal sequence cleavage sites. Nucl Acids Res 1986;14:4683–90.
[50] Sierkstra LN, Verbakel JMA, Verrips CT. Optimisation of a host/vector system for heterologous gene expression by *Hansenula polymorpha*. Curr Genet 1991;19:81–7.
[51] Muller S, Sandal T, Kamp–Hansen P, Dalboge H. Comparison of expression systems in the yeasts *Saccharomyces cerevisiae, Hansenula polymorpha, Klyveromyces lactis, Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Cloning of two novel promoters from *Yarrowia lipolytica*. Yeast 1998;14:1267–83.
[52] Gellissen G, Melber K. Methylotrophic yeast *Hansenula polymorpha* as production organism for recombinant pharmaceuticals. Drug Res 1996;46:943–8.
[53] Lepetic A, Seigelchifer M, Arduino RC, Lazovski J, Nacinovich F, Sturba E, Stambou-lian D. Novel recombinant HB vaccine produced by a high-level *Hansenula polymorpha* yeast system. Clin Infect Dis 1996;23:276.
[54] Gellissen G, Piontek M, Dahlems U, Jenzelewski V, Gavagan JE, DiCosimo R, Anton DL, Janowicz ZA. Recombinant *Hansenula polymorpha* as a biocatalyst: coexpression of the spinach glycolate oxidase (GO) and the *S. cerevisiae* catalase T (CTT1) gene. J Bacteriol 1996;178:4420–8.
[55] Sudbery P, Cox H, Evans L. High level production of glucose oxidase in *Hansenula polymorpha* using the constitutive *PMA1* promoter. In: Diederichs H, Hempel V, Höner CB, editors. *Biotec 96, Forum für Biotechnology*. BioGenTec NRW, Düsseldorf, Germany, 1997. p. 45.
[56] Raschke WC, Neiditch BR, Hendricks M, Cregg JM. Inducible expression of a heterologous protein in *Hansenula polymorpha* using the alcohol oxidase 1 promoter of *Pichia pastoris*. Gene 1996;177:163–7.
[57] Fellinger AJ, Verbakel JM, Veale RA, Sudbery PE, Bom IJ, Overbeeke N, Verrips CT. Expression of the alpha-galactosidase from *Cyamopsis tetragonoloba* (guar) by *Hansenula polymorpha*. Yeast 1991;7:463–73.
[58] Veale RA, Giuseppin ML, van EH, Sudbery PE, Verrips CT. Development of a strain of *Hansenula polymorpha* for the efficient expression of guar alpha-galactosidase. Yeast 1992;8:361–72.

[59] Kang HA, Sohn JH, Choi ES, Chung BH, Yu MH, Rhee SK. Glycosylation of human alpha 1-antitrypsin in *Saccharomyces cerevisiae* and methylotrophic yeasts. Yeast 1998;14:371–81.

[60] Shen SH, Bastien L, Nguyen T, Fung M, Slilaty SN. Synthesis and secretion of hepatitis B middle surface antigen by the methylotrophic yeast *Hansenula polymorpha*. Gene 1989;84:303–9.